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(71) Applicant: CHIRON CORPORATION [US/US]; 4560 Horton Street, Emeryville, CA 94608 (US).

(72) Inventors: RALSTON, Robert, O. ; 52 Long View Court, Danville, CA 94526 (US). MARCUS, Frank ; 43 Remington Court, Danville, CA 94526 (US). THUDIUM, Kent, B. ; 1220 Hampel Street, Oakland, CA 94602 (US). GERVASE, Barbara, A. ; 1001 Cimarron Court, Vallejo, CA 94589 (US). HALL, John, A. ; 725 Racquet Club Circle, Rohnert Park, CA 94928 (US).

(74) Agents: MONROY, Gladys, H. et al.; Morrison & Foerster, 545 Middlefield Road, Suite 200, Menlo Park, CA 94025 (US).

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(54) Title: HEPATITIS C VIRUS ASIALOGLYCOPROTEINS

(57) Abstract

Two Hepatitis C Virus envelope proteins (E1 and E2) are expressed without sialylation. Recombinant expression of these proteins in lower eukaryotes, or in mammalian cells in which terminal glycosylation is blocked, results in recombinant proteins which are more similar to native HCV glycoproteins. When isolated by GNA lectin affinity, the E1 and E2 proteins aggregate into virus-like particles. Cells bearing a mannose receptor or asialoglycoprotein receptor are capable of being infected with HCV, and supporting culturing of the virus.

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HEPATITIS C VIRUS ASIALOGLYCOPROTEINS

Description

Technical Field

This invention relates to the general fields of recombinant protein expression and virology. More particularly, the invention relates to glycoproteins useful for diagnosis, treatment, and prophylaxis of Hepatitis C virus (HCV) infection, and methods for producing such glycoproteins.

Background of the Invention

Non-A, Non-B hepatitis (NANBH) is a transmissible disease (or family of diseases) that is believed to be virally induced, and is distinguishable from other forms of virus-associated liver disease, such as those caused by hepatitis A virus (HAV), hepatitis B virus (HBV), delta hepatitis virus (HDV), cytomegalovirus (CMV) or Epstein-Barr virus (EBV). Epidemiologic evidence suggests that there may be three types of NANBH: the water-borne epidemic type; the blood or needle associated type; and the sporadically occurring community acquired type. The number of causative agents is unknown. However, a new viral species, hepatitis C virus (HCV) has recently been identified as the primary (if not only) cause of blood-borne NANBH (BB-NANBH). See for example PCT WO89/046699. Hepatitis C appears to be the major form of transfusion-associated hepatitis in a number of countries or regions, including the United States, Europe, and Japan. There is also evidence implicating HCV in induction of hepatocellular carcinoma. Thus, a need exists for an effective method for preventing and treating HCV infection.

which is translated from the predicted E1 region of the HCV genome. E2 (envelope protein 2) is a glycoprotein having a molecular weight of about 72 kD which is translated from the predicted NS1 (non-structural protein 1) region of the HCV genome, based on the flaviviral model of HCV. As viral glycoproteins are often highly immunogenic, E1 and E2 are prime candidates for use in immunoassays and therapeutic/prophylactic vaccines.

The discovery that E1 and E2 are not sialylated is significant. The particular form of a protein often dictates which cells may serve as suitable hosts for recombinant expression. Prokaryotes such as E. coli do not glycosylate proteins, and are generally not suitable for production of glycoproteins for use as antigens because glycosylation is often important for full antigenicity, solubility, and stability of the protein. Lower eukaryotes such as yeast and fungi glycosylate proteins, but are generally unable to add terminal sialic acid residues to the carbohydrate complexes. Thus, yeast-derived proteins may be antigenically distinct from their natural (non-recombinant) counterparts. Expression in mammalian cells is preferred for applications in which the antigenicity of the product is important, as the glycosylation of the recombinant protein should closely resemble that of the wild viral proteins.

New evidence indicates that the HCV virus may gain entry to host cells during infection through either the asialoglycoprotein receptor found on hepatocytes, or through the mannose receptor found on hepatic endothelial cells and macrophages (particularly Kupffer cells). Surprisingly, it has been found that the bulk of natural E1 and E2 do not contain terminal sialic acid residues, but are only core-glycosylated. A small fraction additionally contains terminal N-acetylglucosamine. Accordingly, it is an object of the present invention to provide HCV envelope glycoproteins lacking all or substantially all terminal sialic acid residues.

Another aspect of the invention is a method for producing asialo-E1 or E2, under conditions inhibiting addition of terminal sialic acid, e.g., by expression

Modes of Carrying Out The Invention

A. Definitions

The term "asialoglycoprotein" refers to a glycosylated protein which is substantially free of sialic acid moieties. Asialoglycoproteins may be prepared recombinantly, or by purification from cell culture or natural sources. Presently preferred asialoglycoproteins are derived from HCV, preferably the glycoproteins E1 and E2, most preferably recombinant E1 and E2 (rE1 and rE2). A protein is "substantially free" of sialic acid within the scope of this definition if the amount of sialic acid residues does not substantially interfere with binding of the glycoprotein to mannose-binding proteins such as GNA. This degree of sialylation will generally be obtained where less than about 40% of the total N-linked carbohydrate is sialic acid, more preferably less than about 30%, more preferably less than about 20%, more preferably less than about 10%, more preferably less than about 5%, and most preferably less than about 2%.

The term "E1" as used herein refers to a protein or polypeptide expressed within the first 400 amino acids of an HCV polyprotein, sometimes referred to as the E or S protein. In its natural form it is a 35 kD glycoprotein which is found strongly membrane-associated. In most natural HCV strains, the E1 protein is encoded in the viral polyprotein following the C (core) protein. The E1 protein extends from approximately amino acid 192 to about aa383 of the full-length polyprotein. The term "E1" as used herein also includes analogs and truncated mutants which are immunologically crossreactive with natural E1.

The term "E2" as used herein refers to a protein or polypeptide expressed within the first 900 amino acids of an HCV polyprotein, sometimes referred to as the NS1 protein. In its natural form it is a 72 kD glycoprotein which is found strongly membrane-associated. In most natural HCV strains, the E1 protein follows the E1 protein. The E2 protein extends from approximately aa384 to about aa820. The term "E2" as used herein also includes analogs and truncated mutants which are immunologically crossreactive with natural E2.

The term "GNA lectin" refers to Galanthus nivalus agglutinin, a commercially available lectin which binds to mannose-terminated glycoproteins.

A "recombinant" glycoprotein as used herein is a glycoprotein expressed from a recombinant polynucleotide, in which the structural gene encoding the glycoprotein is expressed under the control of regulatory sequences not naturally adjacent to the structural gene, or in which the structural gene is modified. For example, one may form a vector in which the E1 structural gene is placed under control of a functional fragment of the yeast glyceraldehyde-3-phosphate dehydrogenase (GAPDH) promoter. A presently preferred promoter for use in yeast is the hybrid ADH2/GAP promoter described in U.S. Pat. No. 4,880,734, which employs a fragment of the GAPDH promoter in combination with the upstream activation sequence derived from alcohol dehydrogenase 2. Modifications of the structural gene may include substitution of different codons with degenerate codons (e.g., to utilize host-preferred codons, eliminate or generate restriction enzyme cleavage sites, to control hairpin formation, etc.), and substitution, insertion or deletion of a limited number of codons encoding different amino acids (preferably no more than about 10%, more preferably less than about 5% by number of the natural amino acid sequence should be altered), and the like. Similarly, a "recombinant" receptor refers to a receptor protein expressed from a recombinant polynucleotide, in which the structural gene encoding the receptor is expressed under the control of regulatory sequences not naturally adjacent to the structural gene, or in which the structural gene is modified.

The term "isolated polypeptide" refers to a polypeptide which is substantially free of other HCV viral components, particularly polynucleotides. A polypeptide composition is "substantially free" of another component if the weight of the polypeptide in the composition is at least 70% of the weight of the polypeptide and other component combined, more preferably at least about 80%, still more preferably about 90%, and most preferably 95% or greater. For example, a composition containing 100 μg/mL E1 and only 3 μg/mL other HCV components (e.g.,

rier, in the presence or absence of an adjuvant. "Neutralization" refers to an immune response that blocks the infectivity, either partially or fully, of an infectious agent. A "vaccine" is an immunogenic composition capable of eliciting protection against HCV, whether partial or complete, useful for treatment of an individual.

The term "biological liquid" refers to a fluid obtained from an organism, such as serum, plasma, saliva, gastric secretions, mucus, and the like. In general, a biological liquid will be screened for the presence of HCV particles. Some biological fluids are used as a source of other products, such as clotting factors (e.g., Factor VIII:C), serum albumin, growth hormone, and the like. In such cases, it is important that the source biological fluid be free of contamination by virus such as HCV.

B. General Method

The E1 region of the HCV genome is described in EP 388,232 as region "E", while E2 is described as "NS1." The E1 region comprises approximately amino acids 192-383 in the full-length viral polyprotein. The E2 region comprises approximately amino acids 384-820. The complete sequences of prototypes of these proteins (strain HCV-1) are available in the art (see EP 388,232), as are general methods for cloning and expressing the proteins. Both E1 and E2 may be expressed from a polynucleotide encoding the first 850-900 amino acids of the HCV polyprotein: post-translational processing in most eukaryotic host cells cleaves the initial polyprotein into C, E1, and E2. One may truncate the 5' end of the coding region to reduce the amount of C protein produced.

Expression of asialoglycoproteins may be achieved by a number of methods. For example, one may obtain expression in lower eukaryotes (such as yeast) which do not normally add sialic acid residues to glycosylated proteins. In yeast expression systems, it is presently preferred to employ a secretion leader such as the S. cerevisiae α-factor leader, so that the protein is expressed into the culture

which apparently anchors the protein within the endoplasmic reticulum and prevents efficient release. Thus, one may wish to delete portions of the sequence found in one or more of the regions aa170-190, aa260-290 or aa330-380 of E1 (numbering from the beginning of the polyprotein), and aa660-830 of E2 (see for example Figure 20-1 of EP 388,232). It is likely that at least one of these hydrophobic domains forms a transmembrane region which is not essential for antigenicity of the protein, and which may thus be deleted without detrimental effect. The best region to delete may be determined by conducting a small number of deletion experiments within the skill of the ordinary practitioner. Deletion of the hydrophobic 3' end of E2 results in secretion of a portion of the E2 expressed, with sialylation of the secreted protein.

One may use any of a variety of vectors to obtain expression. Lower eukaryotes such as yeast are typically transformed with plasmids using the calcium phosphate precipitation method, or are transfected with a recombinant virus. The vectors may replicate within the host cell independently, or may integrate into the host cell genome. Higher eukaryotes may be transformed with plasmids, but are typically infected with a recombinant virus, for example a recombinant vaccinia virus. Vaccinia is particularly preferred, as infection with vaccinia halts expression of host cell proteins. Presently preferred host cells include HeLa and plasmacytoma cell lines. In the present system, this means that E1 and E2 accumulate as the major glycosylated species in the host ER. As the rE1 and rE2 will be the predominant glycoproteins which are mannose-terminated, they may easily be purified from the cells by using lectins such as Galanthus nivalus agglutinin (GNA) which bind terminal mannose residues.

Proteins which are naturally expressed as mannose-terminated glycoproteins are relatively rare in mammalian physiology. In most cases, a mammalian glycoprotein is mannose-terminated only as a transient intermediate in the glycosylation pathway. The fact that HCV envelope proteins, expressed recombinantly, contain mannose-terminated glycosylation or (to a lesser degree) N-acetyl-

remove mannose-terminated glycoproteins from serum or plasma fractions, thus reducing or eliminating the risk of HCV contamination.

It is presently preferred to isolate E1 and/or E2 asialoglycoproteins from crude cell lysates by incubation with an immobilized mannose-binding protein, particularly a lectin such as ConA or GNA. Cells are lysed, e.g., by mechanical disruption in a hypotonic buffer followed by centrifugation to prepare a post-nuclear lysate, and further centrifuged to obtain a crude microsomal membrane fraction. The crude membrane fraction is subsequently solubilized in a buffer containing a detergent, such as Triton X-100, NP40, or the like. This detergent extract is further clarified of insoluble particulates by centrifugation, and the resulting clarified lysate incubated in a chromatography column comprising an immobilized mannose-binding protein, preferably GNA bound to a solid support such as agarose or Sepharose® for a period of time sufficient for binding, typically 16 to 20 hours. The suspension is then applied to the column until E1/E2 begins to appear in the eluent, then incubated in the column for a period of time sufficient for binding, typically about 12-24 hours. The bound material is then washed with additional buffer containing detergent (e.g., Triton X-100, NP40, or the like), and eluted with mannose to provide purified asialoglycoprotein. On elution, it is preferred to elute only until protein begins to appear in the eluate, at which point elution is halted and the column permitted to equilibrate for 2-3 hours before proceeding with elution of the protein. This is believed to allow sufficient time for the slow off-rate expected of large protein aggregates. In cases wherein E1 and E2 are expressed together in native form (i.e., without truncation of the membrane-binding domain), a substantial fraction of the asialoglycoproteins appear as E1:E2 aggregates. When examined by electron microscopy, a significant portion of these aggregates appear as roughly spherical particles having a diameter of about 40 nm, which is the size expected for intact virus. These particles appear to be self-assembling subviral particles. These aggregates are expected to exhibit a

ally express the receptor, using conditions under which the receptor is maintained, or one may transfect another cell line such as HeLa, CHO, COS, and the like, with a vector providing for expression of the receptor. Cloning of the mannose receptor and its transfection and expression in fibroblasts has been demonstrated by Taylor et al., supra, while transfection and expression of functional ASGR in rat HTC cells was described by McPhaul et al., supra. It is presently preferred to employ an immortalized cell line transfected with one or both recombinant receptors.

Immunogenic compositions can be prepared according to methods known in the art. The present compositions comprise an immunogenic amount of a polypeptide, e.g., E1, E2, or E1/E2 particle compositions, usually combined with a pharmaceutically acceptable carrier, preferably further comprising an adjuvant. If a "cocktail" is desired, a combination of HCV polypeptides, such as, for example, E1 plus E2 antigens, can be mixed together for heightened efficacy. The virus-like particles of E1/E2 aggregates are expected to provide a particularly useful vaccine antigen. Immunogenic compositions may be administered to animals to induce production of antibodies, either to provide a source of antibodies or to induce protective immunity in the animal.

Pharmaceutically acceptable carriers include any carrier that does not itself induce the production of antibodies harmful to the individual receiving the composition. Suitable carriers are typically large, slowly metabolized macromolecules such as proteins, polysaccharides, polylactic acids, polyglycolic acids, polymeric amino acids, amino acid copolymers; and inactive virus particles. Such carriers are well known to those of ordinary skill in the art.

Preferred adjuvants to enhance effectiveness of the composition include, but are not limited to: aluminum hydroxide (alum), N-acetyl-muramyl-L-threonyl-D-isoglutamine (thr-MDP) as found in U.S. Patent No. 4,606,918, N-acetyl-normuramyl-L-alanyl-D-isoglutamine (nor-MDP), N-acetylmuramyl-L-alanyl-D-isoglutaminyl-L-alanine-2-(1'-2'-dipalmitoyl-sn-glycero-3-hydroxyphosphoryloxy)-

surface antigen (see European Patent Application 174,444). In this use, the E1/E2 aggregates provide an immunogenic carrier capable of stimulating an immune response to haptens or antigens conjugated to the aggregate. The antigen may be conjugated either by conventional chemical methods, or may be cloned into the gene encoding E1 and/or E2 at a location corresponding to a hydrophilic region of the protein.

The immunogenic compositions are conventionally administered parenterally, typically by injection, for example, subcutaneously or intramuscularly. Additional formulations suitable for other modes of administration include oral formulations and suppositories. Dosage treatment may be a single dose schedule or a multiple dose schedule. The vaccine may be administered in conjunction with other immunoregulatory agents.

C. Examples

The examples presented below are provided as a further guide to the practitioner of ordinary skill in the art, and are not to be construed as limiting the invention in any way.

Example 1

(Cloning and Expression)

(A) Vectors were constructed from plasmids containing the 5' portion of the HCV genome, as described in EP 318,216 and EP 388,232. Cassette HCV(S/B) contains a StuI-BglII DNA fragment encoding the 5' end of the polyprotein from Met_1 up to Leu_{906}, beginning at nucleotide -63 relative to Met_1. This includes the core protein (C), the E1 protein (also sometimes referred to as S), the E2 protein (also referred to as NS1), and a 5' portion of the NS2a region. Upon expression of the construct, the individual C, E1 and E2 proteins are produced by proteolytic processing.

pellets were resuspended at a final concentration of 2×10^7 cells/mL in additional culture medium (Joklik modified MEM Spinner medium +5% horse serum and Gentamicin) ("spinner medium"). Sonicated crude vv/SC59-HCV virus stock was added at a multiplicity of infection of 8 pfu/cell, and the mixture stirred at 37°C for 30 minutes. The infected cells were then transferred to a spinner flask containing 8 L spinner medium and incubated for 3 days at 37°C.

The cultured cells were then collected by centrifugation, and the pellets resuspended in buffer (10 mM Tris-HCl, pH 9.0, 152 mL). The cells were then homogenized using a 40 mL Dounce Homogenizer (50 strokes), and the nuclei pelleted by centrifugation (5 minutes, 1600 rpm, 4°C, JA-20 rotor). The nuclear pellets were resuspended in Tris buffer (24 mL), rehomogenized, and pelleted again, pooling all supernatants.

The pooled lysate was divided into 10 mL aliquots and sonicated 3×30 minutes in a cuphorn sonicator at medium power. The sonicated lysate (15 mL) was layered onto 17 mL sucrose cushions (36%) in SW28 centrifuge tubes, and centrifuged at 13,500 rpm for 80 minutes at 4°C to pellet the virus. The virus pellet was resuspended in 1 mL of Tris buffer (1 mM Tris HCl, pH 9.0) and frozen at -80°C.

Example 2

(Comparison of In Vitro and In Vivo Products)

(A) E1 and E2 were expressed both in vitro and in vivo and ^{35}S-Met labeled using the vectors described in Example 1 above. BSC-40 and HeLa cells were infected with the rVV vectors for in vivo expression. Both the medium and the cell lysates were examined for recombinant proteins. The products were immunoprecipitated using human HCV immune serum, while in vitro proteins were analyzed directly. The resulting proteins were analyzed by SDS-PAGE.

The reticulocyte expression system (pGEM3Z with HCV(S/B) or HCV(A/B)) produced C, E1 and E2 proteins having molecular weights of approxi-

ture medium. A 25 kD component was observed, but appears to be specific to vaccinia-infected cells.

Example 3

(Purification Using Lectin)

(A) HeLa S3 cells were inoculated with purified high-titer vv/SC59-HCV virus stock at a multiplicity of infection of 5 pfu/cell, and the mixture stirred at 37°C for 30 minutes. The infected cells were then transferred to a spinner flask containing 8 L spinner medium and incubated for 3 days at 37°C. The cells were collected again by centrifugation and resuspended in hypotonic buffer (20 mM HEPES, 10 mM NaCl, 1 mM $MgCl_2$, 120 mL) on ice. The cells were then homogenized by Dounce Homogenizer (50 strokes), and the nuclei pelleted by centrifugation (5 minutes, 1600 rpm, 4°C, JA-20 rotor). The pellets were pooled, resuspended in 48 mL hypotonic buffer, rehomogenized, recentrifuged, pooled again, and frozen at -80°C.

The frozen supernatants were then thawed, and the microsomal membrane fraction of the post-nuclear lysate isolated by centrifuging for 20 minutes in a JA-20 rotor at 13,500 rpm at 4°C. The supernatant was removed by aspiration.

The pellets were taken up in 96 mL detergent buffer (20 mM Tris-HCl, 100 mM NaCl, 1 mM EDTA, 1 mM DDT, 0.5% Triton X-100, pH 7.5) and homogenized (50 strokes). The product was clarified by centrifugation for 20 minutes at 13,500 rpm, 4°C, and the supernatants collected.

A GNA-agarose column (1 cm × 3 cm, 3 mg GNA/mL beads, 6 mL bed volume, Vector Labs, Burlingame, CA) was pre-equilibrated with detergent buffer. The supernatant sample was applied to the column with recirculation at a flow rate of 1 mL/min for 16-20 hours at 4°C. The column was then washed with detergent buffer.

The purified E1/E2 proteins were eluted with α-D-mannoside (0.9 M in detergent buffer) at a flow rate of 0.5 mL/minute. Elution was halted at the

(A) The GNA lectin-purified material prepared as described in Example 3 (0.5-0.8 mL) was diluted 10× with buffer A (20 mM Tris-Cl buffer, pH 8.0, 1 mM EDTA), and applied to a 1.8 × 1.5 cm column of Fractogel EMD DEAE-650 (EM Separations, Gibbstown, New Jersey, cat. no. 16883) equilibrated in buffer A. The protein fraction containing E1/E2 was eluted with the same buffer at a flow rate of 0.2 mL/minute, and 1 mL fractions collected. Fractions containing E1 and E2 (determined by SDS-PAGE) were pooled and stored at -80°C.

(B) The material purified in part (A) above has a purity of 60-80%, as estimated by SDS-PAGE. The identification of the putative E1 and E2 bands was confirmed by N-terminal sequence analysis after using a transfer technique. For the purpose, the fractogel-DEAE purified E1/E2 material was reduced by addition of Laemmli buffer (pH 6.8, 0.06 M Tris-Cl, 2.3% SDS, 10% glycerol, 0.72 M β-mercaptoethanol) and boiled for 3 minutes. The sample was then loaded onto a 10% polyacrylamide gel. After SDS-PAGE, the protein was transferred to a polyvinylidene difluoride (PVDF) 0.2 μm membrane (Bio-Rad Laboratories, Richmond, CA). The respective putative E1 and E2 protein bands were excised from the blot and subjected to N-terminal amino acid analysis, although no special care was taken to prevent amino-terminal blockage during preparation of the material. The first 15 cycles revealed that the E1 sample had a sequence Tyr-Gln-Val-Arg-X-Ser-Thr-Gly-X-Tyr-His-Val-X-Asn-Asp, while the sequence of E2 was Thr-His-Val-Thr-Gly-X-X-Ala-Gly-His-X-Val-X-Gly-Phe. This amino acid sequence data is in agreement with that expected from the corresponding DNA sequences.

The E1/E2 product purified above by fractogel-DEAE chromatography is believed to be aggregated as evidenced by the fact that a large amount of E1 and E2 coelutes in the void volume region of a gel permeation chromatographic Bio-Sil TSK-4000 SW column. This indicates that under native conditions a significant amount of the E1/E2 complex has a molecular weight of at least 800 kD. E1/E2 material having a molecular weight of about 650 kD was also observed.

7. The method of claim 6, wherein said condition inhibiting sialylation comprises expression of E1 or E2 at a rate sufficient to inhibit transport of glycoproteins from the endoplasmic reticulum to the golgi.

8. The method of claim 6, wherein said conditions inhibiting sialylation comprise:

presence of a sufficient amount of a calcium modulator to cause release of proteins within the host cell's endoplasmic reticulum.

9. A method for purifying HCV asialoglycoproteins, which method comprises:

contacting a composition containing HCV asialoglycoproteins with a mannose-binding protein; and

isolating the portion of the composition which binds to said mannose-binding protein.

10. The method of claim 9, wherein said mannose-binding protein is a lectin selected from the group consisting of ConA and GNA.

11. The method of claim 9, wherein:

said contacting comprises incubation of said composition containing HCV asialoglycoproteins in a column comprising a mannose-binding lectin immobilized on a support, for a period of at least one hour; and

said isolating comprises eluting said HCV asialoglycoproteins with mannose.

12. An assay kit for detecting the presence of HCV asialoglycoproteins, said kit comprising:

a solid support;

18. A method of inducing an immune response in an animal, which method comprises:

providing a vaccine composition comprising an effective amount of an HCV asialoglycoprotein in a pharmaceutically acceptable vehicle;

administering said vaccine composition to said animal.

19. An HCV asialoglycoprotein composition, comprising: purified HCV E1/E2 asialoglycoprotein aggregate.

20. A method for propagating HCV in cell culture comprising:

(a) providing a cell that expresses a receptor selected from the group consisting of the mannose receptor and the asialoglycoprotein receptor;

(b) infecting the cell with HCV; and

(c) culturing the infected cell.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US91/08272

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

ICP(5): C07K 9/00, 15/00; C12N 15/00; A61K 37/10
US CL: 435/69.9, 172.3; 530/322,395; 514/8

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
US CL:	435/69.9, 172.3; 530/322,395; 514/8

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, CAS ON LINE

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	EP, A, 0,388,232 (HOUGHTON ET AL.), 19 September 1990, see the entire document, particularly claims 6-12.	1-7
Y	EP, A, 0,318,216 (HOUGHTON ET AL.) 31 May 1989, see the entire document, particularly claims 12-16, 20, 27, 28.	1-7,19
Y	EP, A, 0,320,267 (DINKA) 14 June 1989, see the entire document, particularly the abstract.	9-11

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
28 January 1992	28 FEB 1992
International Searching Authority	**Signature of Authorized Officer**
ISA/US	Fatemeh Moezie FATEMEH MOEZIE

PART VI. Observations Where Unity Of Invention IS Lacking

Restriction of the following inventions is required:

I. Claims 1-11 and 19, drawn to an HCV asialoglycoprotein and method of producing the protein, classified in Class 530, subclass 395, for example.

II. Claim 12, drawn to an assay kit, classified in Class 422, subclass 61, for example.

III. Claims 14-16, drawn to a transformed cell, classified in Class 435, subclass 240.2, for example.

IV. Claims 13 and 17, drawn to a method of determining exposure to or infection by HCV and a method of eliminating the presence of HCV, both in-vitro, classified in Class 436, subclass 63, for example.

V. Claim 18, drawn to a method of treatment, classified in Class 514, subclass 12, for example.

VI. Claim 20, drawn to a method of propagating HCV in cell culture, classified in Class 435, subclass 41, for example.

WO 92/06734(3)
C07K14/18-[M07K201:02]-[M07K203:00]-
[M07K207:[illegible]]-

0556292

Application Number
EP 92 90 0091

- 1- *-

C07K14/18

EUROPEAN SEARCH REPORT

DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document with indication, where appropriate, of relevant passages	Relevant to claim	CLASSIFICATION OF THE APPLICATION (Int.Cl.5)
X,P	WO-A-91 15771 (CHIRON CORPORATION) * examples 3,6 * -----	16-21	C07K9/00 C07K15/00 C12N15/00 A61K37/10
			TECHNICAL FIELDS SEARCHED (Int.Cl.5)
			C07K C12N A61K G01N

The supplementary search report has been drawn up for the claims attached hereto.

1

Place of search	Date of completion of the search	Examiner
THE HAGUE	17 November 1994	Skelly, J

CATEGORY OF CITED DOCUMENTS

X : particularly relevant if taken alone
Y : particularly relevant if combined with another document of the same category
A : technological background
O : non-written disclosure
P : intermediate document

T : theory or principle underlying the invention
E : earlier patent document, but published on, or after the filing date
D : document cited in the application
L : document cited for other reasons

& : member of the same patent family, corresponding document

WHAT IS CLAIMED:

1. A method for producing HCV asialoglycoproteins suitable for use in a vaccine or immunoassay, which method comprises:

growing a lower eukaryote transformed with a structural gene encoding an HCV asialoglycoprotein selected from the group consisting of E1 and E2 in a suitable culture medium;

causing expression of said structural gene; and

isolating said HCV asialoglycoprotein from said cell culture by contacting said HCV asialoglycoprotein with a mannose-binding protein specific for mannose-terminated glycoproteins, and isolating the portion of the composition which binds to said mannose-binding protein.

2. The method of claim 1, wherein said lower eukaryote is yeast.

3. A method for producing HCV asialoglycoproteins suitable for use in a vaccine or immunoassay, which method comprises:

growing a mammalian host cell transformed with a structural gene encoding an HCV asialoglycoprotein selected from the group consisting of E1 and E2 in a suitable culture medium;

causing expression of said structural gene under conditions inhibiting sialylation; and

isolating said HCV asialoglycoprotein from said cell culture by contacting said HCV asialoglycoprotein with a mannose-binding protein specific for mannose-terminated glycoproteins.

4. The method of claim 3, wherein said conditions inhibiting sialylation comprises expression of E1 or E2 at a rate sufficient to inhibit transport of glycoproteins from the endoplasmic reticulum to the golgi.

5. The method of claim 3, wherein said conditions inhibiting sialylation comprise:

the presence of a sufficient amount of a calcium modulator to cause release of proteins within the host cell's endoplasmic reticulum.

6. A method for purifying HCV asialoglycoproteins, which method comprises:

contacting a composition containing HCV asialoglycoproteins with a mannose-binding protein specific for mannose-terminated glycoproteins; and

isolating the portion of the composition which binds to said mannose-binding protein.

7. The method of claim 6, wherein said mannose-binding protein is a lectin.

8. The method of claim 7, wherein said mannose-binding lectin is GNA.

9. The method of claim 6, wherein:

said contacting comprises incubation of said composition containing HCV asialoglycoproteins in a column comprising a mannose-binding lectin immobilized on a support, for a period of at least one hour; and

said isolating comprises eluting said HCV asialoglycoproteins with mannose.

10. An assay kit for detecting the presence of HCV asialoglycoproteins, said kit comprising:

a solid support;

a mannose-binding protein specific for mannose-terminated glycoproteins; and

an antibody specific for said HCV asialoglycoprotein;

wherein one of said antibody and said lectin is bound to said solid support.

11. In a method for determining exposure to or infection by HCV, the method wherein any HCV within a sample of body fluid is concentrated by contact with a mannose-binding protein prior to assay, wherein the mannose-binding protein is specific for mannose-terminated glycoproteins.

12. A method for reducing or eliminating the presence of HCV in plasma, serum, or other biological liquids which method comprises:

contacting said biological liquid with a mannose-binding protein specific for mannose-terminated glycoproteins; and

separating said biological liquid from said mannose-binding protein.

13. A method of inducing an immune response in an animal, which method comprises:

providing a vaccine composition comprising an effective amount of an HCV asialoglyocoprotein in a pharmaceutically effective vehicle, wherein said HCV asialoglycoprotein is E1 or E2.

14. An HCV asialoglycoprotein composition, comprising purified HCV E1/E2 asialoglycoprotein aggregate.

15. An HCV asialoglycoprotein composition suitable for use in the preparation of a vaccine, comprising purified HCV E1/E2 aggregate.

16. An HCV asialoglycoprotein suitable for use in the preparation of a vaccine or an immunoassay reagent wherein the asialoglycoprotein comprises an amino acid sequence from the E1 or E2 domain of HCV.

17. An HCV asialoglycoprotein according to claim 16, wherein the asialoglycoprotein is E1 or E2.

18. An HCV asialoglycoprotein purified by the method of claim 6, or claim 7, or claim 8.

19. An HCV asialoglycoprotein according to claim 18, wherein the said asialoglycoprotein is E1 or E2.

20. An immunoassay reagent comprising an HCV asialoglycoprotein according to claim 16 bound to a suitable support.

21. An immunoassay reagent comprising an HCV asialoglycoprotein according to claim 16 in combination with a suitable label.